

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
6 May 2004 (06.05.2004)

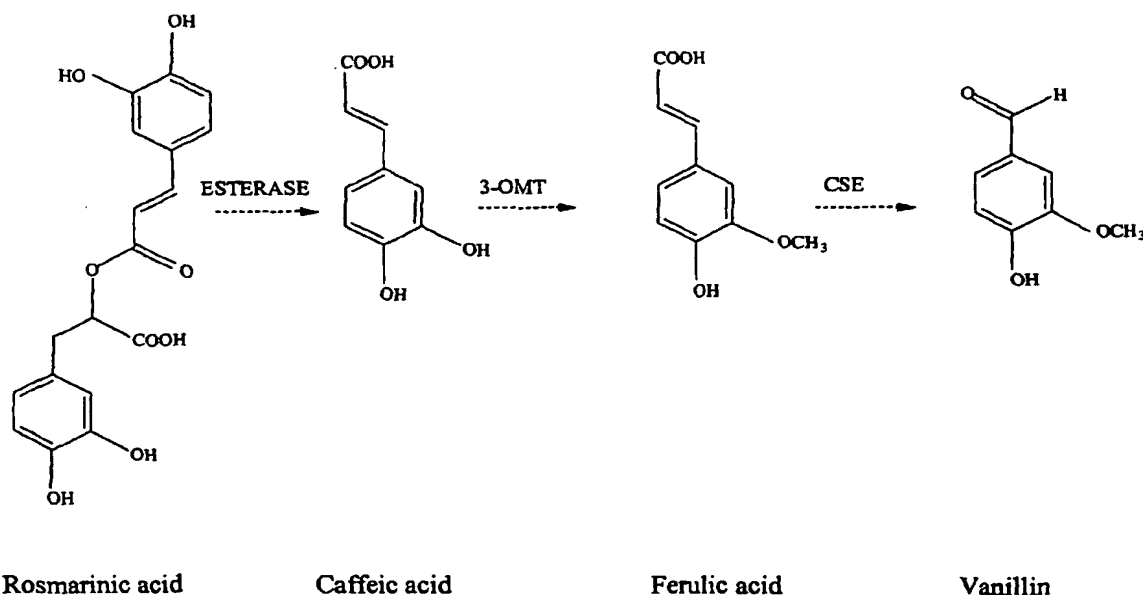
PCT

(10) International Publication Number
WO 2004/036979 A2

- (51) International Patent Classification⁷: **A01H** (74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn LLP, One Liberty Place - 46th Floor, Philadelphia, PA 19103 (US).
- (21) International Application Number: PCT/US2003/034011
- (22) International Filing Date: 23 October 2003 (23.10.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/412,649 23 October 2002 (23.10.2002) US
- (71) Applicant (for all designated States except US): **RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY** [US/US]; Old Queens, Somerset Street, New Brunswick, NJ 08903 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **HAVKIN-FRENKEL, Daphna** [US/US]; 772 Cranbury Crossroad, North Brunswick, NJ 08902-2204 (US). **ZYLSTRA, Gerben, J.** [US/US]; 32 Lake Drive, P.O. Box 632, Roosevelt, NJ 08555-0632 (US). **FRENKEL, Chaim** [US/US]; 772 Cranbury Crossroad, North Brunswick, NJ 08902-2204 (US). **BELANGER, Faith** [US/US]; 3 Agate Road, East Brunswick, NJ 08816 (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished upon receipt of that report

[Continued on next page]

(54) Title: PRODUCTION OF VANILLIN IN MICROBIAL CELLS



(57) Abstract: Transgenic microorganisms that produce vanillin when provided with caffeic acid or an esterified or other derivative thereof are disclosed. The organisms are transformed with expressible nucleic acid sequences encoding (1) a 3-O-methyltransferase, preferably from a plant source, which converts caffeic acid to ferulic acid and (2) either a eukaryotic (preferably plant) non-oxidative chain-shortening enzyme or a bacterial CoA ligase and enoyl-CoA hydratase/lyase enzymatic system, either of which converts ferulic acid to vanillin. Methods of making vanillin in the transgenic microorganisms are also disclosed.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PRODUCTION OF VANILLIN IN MICROBIAL CELLS

Benefit is claimed of U.S. Provisional Application No. 60/412,649, filed October 23, 2002, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of microbial genetic engineering to produce high-value food and nutraceutical substances. In particular, this invention provides novel transgenic microbial cells that produce vanillin.

BACKGROUND OF THE INVENTION

Various patents and publications are referred to throughout the specification. Each of these is incorporated by reference herein in its entirety:

Vanillin is the major principle flavor ingredient in vanilla extract and is also noted as a nutraceutical because of its anti-oxidant and antimicrobial properties. Vanillin can be used as a masking agent for undesirable flavors of other nutraceuticals. Vanilla extract is obtained from cured vanilla beans, the bean-like pod produced by *Vanilla planifolia*, a tropical climbing orchid.

Vanilla extract is widely used as a flavor by the food and beverage industry, and is used increasingly in perfumes. Because of the ever-increasing demand for natural food ingredients, natural vanilla extract produced from vanilla beans is presently the most desirable form of vanilla. The areas of the world capable of supporting vanilla cultivation are limited, due to its requirement for a warm, moist and tropical climate with frequent, but not excessive, rain and moderate sunlight. The primary growing region for vanilla is around the Indian Ocean, in Madagascar, Comoros, Reunion and Indonesia.

The production of vanilla beans is a lengthy process that is highly dependent on suitable soil and weather conditions. Beans (pod-like fruit) are produced after 4-5 years of cultivation. Flowers must be hand-pollinated, and fruit production takes about 8-10 months.

The characteristic flavor and aroma develops in the fruit after a process called "curing," lasting an additional 3-6 months. For a complete review of the vanilla growing and curing process, see D. Havkin Frenkel & R. Dorn, "Vanilla," Chapter 4 in Spices: Flavor Chemistry and Antioxidant Properties, (Eds. Risch & Ho), American Chemical Society, Washington, 1997.

Vanillin is also produced chemically by molecular breakage of curcumin, eugenol or piperin. However, vanillin produced by this method can be labeled as a natural flavor only in non-vanilla flavors. Vanillin chemically synthesized from guaiacol is consumed at a rate of about 2,500 tons per year in the United States for the food and beverage industry. Though less expensive than natural vanillin, vanillin produced by chemical synthesis or breakage can be undesirable due to the market's current preference for natural food ingredients.

Interest has focused recently on plant cell and tissue culture as an approach to control quality and yield of vanilla production and to solve some of the agronomic problems associated with growing vanilla. Another possible means for producing vanillin is through the use of microorganisms engineered to possess the requisite complement of vanillin biosynthetic enzymes.

Several C₆-C₃ source compounds, mostly eugenol and ferulic acid, are currently in use in conjunction with fermentation technologies, for the biotechnological production of vanillin (Benz, 1996, Biotechnological production of vanillin. In: AJ Taylor and DS Mottram, Eds, Flavour science - Recent Development, The Royal Society of Chemistry, Cambridge, UK, pp 111-117). Eugenol, a major aromatic constituent in clove oil, is converted by a *Pseudomonas* strain to ferulic acid through successive steps entailing formation of coniferyl alcohol, coniferyl aldehyde and, finally, ferulic acid. Ferulic acid is present also in cereal crops where the compound is esterified to arabinose moieties comprising around 0.4 to 3.0 % of the cell wall material (Walton et al., 2000, Curr. Op. Biotechnol. 11: 490-496). Ferulic acid may be released from the cell wall matrix with the use of strong alkali or by enzymatic cleavage of the wall material using cinnamoyl esterase in combination with cell wall hydrolyzing enzymes (Williamson et al., 1998, Microbiology 144: 2011-2023). Such processes are expensive and time consuming, and can require specialized equipment.

Moreover, bioconversion of ferulic acid to produce vanillin, as opposed to undesired by-products such as vanillic acid, heretofore has not been a straightforward process. Although ferulate is readily metabolized by various microbial systems, the end product is mostly vanillic acid (Dignum & Verpoorte, 2001, Food Rev. Int. 17: 199-219).

It appears that the mode of degradation of a three-carbon side chain of a hydroxycinnamic acid derivative, eugenol or ferulic acid for instance, to a single carbon moiety determines the metabolic fate of phenylpropanoid compounds. There are several reports on the *in vitro* chain shortening-catalyzed degradation of C₆-C₃ to C₆-C₁ compounds, such as benzoic acids and aldehydes, from hydroxycinnamic acids. One study on the synthesis of 4-hydroxybenzoate in *L. erythrorhizon* indicates that the pathway entails oxidation and cleavage of 4-coumaroyl CoA to 4-hydroxybenzoyl CoA and acetyl CoA in a thiolase type reaction with requirement for NAD (Löscher and Heide, 1994, Plant Physiol 106: 271-279). This mode of enzyme action, involving oxidative chain shortening, may account for the formation of vanillic acid as an oxidative cleavage product from ferulic acid, instead of the sought-after vanillin.

Microorganisms capable of utilizing abundant and inexpensive starting materials to produce vanillin in a straightforward manner, without unwanted by-products are currently not available. Thus, a need exists for their creation and development.

SUMMARY OF THE INVENTION

The present invention features a transgenic microorganism that produces vanillin when provided with caffeic acid or derivative thereof of esterified coumaric acid. The organism is transformed with expressible nucleic acid sequences encoding (1) a 3-O-methyltransferase, preferably from a plant source, which converts caffeic acid to ferulic acid and (2) either a eukaryotic (preferably plant) non-oxidative chain-shortening enzyme or a bacterial CoA ligase and enoyl-CoA hydratase/lyase enzymatic system, either of which converts ferulic acid to vanillin. In one embodiment, the microorganism comprises, naturally or *via* recombinant means, an expressible nucleic acid molecule encoding an esterase that converts caffeic acid esters (e.g., cichoric acid, rosmarinic acid or chlorogenic acid) to caffeic acid.

In one embodiment, the transgenic microorganism is a procaryote, such as *E. coli*, *Pseudomonas* or any other prokaryotic microorganism that can be transformed and used for expression of foreign proteins. In another embodiment, the transgenic microorganism is a eucaryote, such as the yeasts *Saccharomyces cerevisiae* or, in a preferred embodiment, *Pichia pastoris*. The microorganism preferably one that does not degrade or further metabolize vanillin, once it is produced.

The present invention also features a method for producing vanillin, which comprises: (a) providing a transgenic organism that produces vanillin when provided with

caffeic acid or an esterified derivative thereof, as described above; (b) culturing the transgenic organism in the presence of the caffeic acid or derivative thereof, under conditions whereby the transgenic organism produces vanillin; and (c) recovering the vanillin from the culture.

5 Another aspect of the invention features an O-methyltransferase from *Vanilla planifolia* that catalyzes methylation of substrates selected from the group consisting of 5-OH-ferulic acid ethyl ester, caffeic acid ethyl ester, caffeoyl aldehyde, 5-OH-coniferaldehyde, 5-OH-ferulic acid, 3,4-dihydroxybenzaldehyde and caffeic acid. In one embodiment, the enzyme has an amino acid sequence at least 90% identical to SEQ ID NO:2, and more specifically comprises amino acid SEQ ID NO:2.

10 Also featured is an isolated nucleic acid molecule that encodes the O-methyltransferase described above. In one embodiment, the nucleic acid encodes a polypeptide having an amino acid sequence at least 90% identical to SEQ ID NO:2 and more specifically encodes a polypeptide having SEQ ID NO:2. In an exemplary embodiment, the nucleic acid molecule has a sequence comprising SEQ ID NO:1.

15 Additional features and advantages of the present invention will be understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

20 **Figure 1.** Schematic diagram showing the biotransformation of cichoric acid to vanillin.

Figure 2. Schematic diagram showing the biotransformation of rosmarinic acid to vanillin.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. Definitions

25 Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

30 With reference to nucleic acid molecules the term "isolated nucleic acid" or "isolated polynucleotide" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a

procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule. With respect to RNA molecule, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

The term "enzyme" refers to a protein having enzymatic activity. As used herein, the term enzyme may refer to the singular or plural, in instances where two or more enzymes form an enzymatic system to convert one substance into another.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library. With respect to antibodies, the term, "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

"Variant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide.

Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be naturally occurring, such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variations that do not materially affect the nature of the protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "identity" or "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

"Identity" and "similarity" can be readily calculated by known methods. Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids and thus define the differences. In preferred methodologies, the BLAST programs (NCBI) and parameters used therein are

employed, and the DNASTar system (Madison, WI) is used to align sequence fragments of genomic DNA sequences. However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

With respect to single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site

(conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or
5 expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA" or "transgene". Such a nucleic acid construct may contain a
10 coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily
15 detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the
20 mammalian genomic DNA in the genome of the source organism. In another example, a heterologous region is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein. The
25 term "DNA construct", as defined above, is also used to refer to a heterologous region, particularly one constructed for use in transformation of a cell.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and
30 mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of

daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

The following sections set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for the purpose of illustration, and is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) or Ausubel et al. (eds), Current Protocols in Molecular Biology, John Wiley & Sons (2003) are used.

II. Description

In an effort to develop alternative methods for producing high quality vanillin in an economically feasible way, the inventors have devised a biotransformative pathway that can be engineered into selected species of microorganisms, which results in production of vanillin from a readily available and inexpensive starting material, which is caffeic acid or a substance that readily produces caffeic acid, such as cichoric acid, rosmarinic acid or chlorogenic acid (sometimes referred to collectively herein as "caffeic acid derivatives").

Representative schemes for vanillin biosynthesis in accordance with the invention are outlined in Figures 1 and 2. The schemes indicate that caffeic acid, obtained in the native form or by hydrolysis of caffeic acid esters, is methylated to ferulic acid by 3-O-methyltransferase, a readily available gene product, which has also been cloned from *V. planifolia* itself. In a preferred embodiment of the present invention, ferulic acid is thereafter converted in one-step process to vanillin by the action of a non-oxidative chain-shortening enzyme, exemplified by the *V. planifolia* 4-hydroxybenzaldehyde synthase (4-HBS) disclosed in U.S. Published Application No. 2003/0070188 A1 (April 2003) to Havkin-Frenkel et al. An alternative embodiment uses bacterial enzymes in a two-step non-oxidative process to convert ferulic acid to vanillin.

Advantageous to the present invention, caffeic acid or caffeic acid derivatives are abundant in several plant species. These compounds are readily hydrolyzed by esterase action, resulting in the release of caffeic acid (Nüsslein et al. 2000, J. Nat. Prod. 63: 1615-1618). Hence, hydrolytically produced caffeic acid, combined with the content of native caffeic acid, can yield 10 to 15% free caffeic acid on dry weight basis. Because caffeic acid or caffeic acid derivatives are present in plant tissues in a free form and because these compounds are readily extracted (e.g., by ethanol), these materials offer an important

advantage as source compounds. By comparison, the use of ferulic acid itself as source material is predicated on enzymatic release of the compound from its bound form to cell walls. This process is complex, leading to impurities and, importantly, is costly. The method of the present invention averts these problems by making use of abundant and readily extractable caffeic acid or caffeic acid derivatives in plant tissues and by a direct methylation of caffeic acid to form ferulic acid. The subsequent conversion of ferulate to vanillin by a non-oxidative chain shortening enzyme also avoids the inadvertent conversion of C₆-C₃ compound to vanillic acid or other unintended end products; a problem encountered in other production systems.

Natural sources for caffeic acid and derivatives thereof include, but are not limited to, *Echinacea spp.* and other species in the mint family, and may further include any plant species that contain the compounds. In a preferred embodiment, cichoric acid is obtained from *Echinacea spp.* Table 1 shows other plant sources of caffeic acid or its derivatives. In addition to those listed in Table 1, plant species that contain caffeic acid or derivatives suitable for use in the present invention include, but are not limited to, liquorice (*Glycyrrhiza glabra*, *G. inflata*, *G. uralensis*), oregano (*Origanum compactum* and other species), sage (*Salvia fruticosa*), carrot (*Daucus carota*), fennel (*Foeniculum vulgare*) and artichoke (*Cynara cardunculus*).

Table 1. Abundance of Caffeic acid and Caffeic acid derivative in some plant species

| Plant Species | Caffeic Acid | Rosmarinic ¹ Acid | Chicoric ² Acid |
|--------------------------------|--------------|------------------------------|----------------------------|
| % Dry Weight ³ | | | |
| <i>Ocimum basilicum</i> | 0.5-2.5 | 5.0-8.0 | |
| <i>Agastache sp.</i> | 1.0-2.0 | 8.0-10.0 | |
| <i>Echinacea purpurea</i> | 3.6 | | 16.7 |
| <i>Mentha sp.</i> | 1.0-2.0 | 2.0-15.0 | |
| <i>Rosmarinus officianalis</i> | 0.1-2.0 | 4.0-10.0 | |

¹ Cinnamic acid, 3,4-dihydroxy-, 2-ester with 3-(3,4-dihydroxyphenyl) lactic acid

² Tartaric acid, bis (3,4-dihydroxycinnamate)

³ Value were obtained by HPLC analysis.

The biotransformation of cichoric acid to vanillin is shown schematically in Figure 1. A similar biotransformation is accomplished using rosmarinic acid, as shown in Figure 2. Cichoric acid, rosmarinic acid and chlorogenic acid (5-caffeoylquinic acid) are all esters of

caffeic acid, and can be converted to caffeic acid in a similar manner. Other caffeic acid esters that can be utilized as caffeic acid sources include, but are not limited to, 1-caffeoylquinic acid and 1,3-dicaffeoylquinic acid (cynarin). Hydrolysis of caffeic acid esters can be accomplished with heat, pressure and mild alkaline solution, or enzymatically by esterases, which is a preferred embodiment. Esterases suitable for catalyzing the conversion of caffeic acid esters to caffeic acid are known in the art and are present in plants, animals and many microorganisms. In the latter instance, therefore, the esterases often need not be engineered into such microorganisms because they exist there naturally. In a preferred embodiment, a microorganism naturally capable of producing caffeic acid from esters thereof is utilized in the present invention.

Caffeic acid is methylated to produce ferulic acid, using a 3-O-methyltransferase obtainable from numerous plant sources, among other organisms. This enzyme catalyzes a methylation at position 3 on the ring (and may also methylate position 5 if it is hydroxylated). Examples of suitable 3-methyltransferases include, but are not limited to (GenBank Accession Numbers follow each listed source organism): *Catharanthus roseus*, AY028439; *Clarkia breweri*, AF006009; *Coffea canephora*, AF454631; *Eucalyptus gunnii*, X74814; *Festuca arundinacea*, AF153825; *Hordeum vulgare*, U54767; *Hordeum vulgare*, AB086416; *Lolium perenne*, AF010291; *Medicago sativa*, M63853; *Nicotiana tabacum* class I, X74452; *Nicotiana tabacum* class II, X74452; *Ocimum basilicum*, AF154918; *Populus tremuloides*, X62096; *Prunus amygdalus*, X83217; *Saccharum officinarum*, AJ231133; *Sorghum bicolor*, AY217766; *Thalictrum tuberosum*, AF064696; *Triticum aestivum*, AY226581; and *Zea mays*, M73235. Nucleic acid and deduced amino acid sequences set forth in the aforementioned Accessions are each incorporated by reference herein in their entireties. Preferred for use is the 3-O-methyltransferase from *Medicago sativa* (alfalfa). Also preferred for use is the 3-O-methyltransferase from *Vanilla planifolia*. A cDNA sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of this enzyme are shown in the Sequence Listing that forms part of this document.

In one embodiment, ferulic acid is converted to vanillin using a eukaryotic non-oxidative-chain shortening enzyme, such as the plant-derived 4-HBS described in U.S. Published Application No. 2003/0070188 A1 to Havkin-Frenkel et al. (2003). Any similar eukaryotic aldehyde synthase that acts as a non-oxidative chain-shortening enzyme may also be utilized. Conversion of ferulic acid to vanillin by non-oxidative means offers the advantage of reducing or eliminating formation of undesired vanillic acid, as discussed above.

In an alternative embodiment, ferulic acid is converted to vanillin using a bacterial chain shortening enzyme system, enoyl-SCoA hydratase/lyase (Gasson et al., 1998, J. Biol. Chem. 273: 4163-4170). The bacterial process is a two-step enzymatic process, involving first a CoA ligase (an enzyme found in eukaryotes, see, e.g., Gross et al., 1973, FEBS Lett. 31: 283-287, as well as bacteria) to activate ferulate to the CoA derivative and then a hydratase/lyase to enzymatically cleave the double bond, releasing vanillin and acetyl-CoA. This enzyme system has been demonstrated to catalyze the conversion of ferulic acid to vanillin. It is found in a number of bacteria, including but not limited to *Pseudomonas fluorescens* (Civolani et al., 2000, Appl Environ Microbiol. 66: 2311-2317; Narbad and Gasson, 1998, Microbiology 144: 1397-1405), *Pseudomonas putida* (Venturi et al., 1998, Microbiology 144: 965-973), other *Pseudomonas* species (Overhage et al., 1999, Appl Microbiol Biotechnol. 52: 820-828; Overhage and Steinbuchel, 1999, Appl Environ Microbiol. 65: 4837-47) and *Nocardia spp.* (Li and Rosazza, 2000, Appl. Environ. Microbiol. 66: 684-687).

In certain instances, enoyl-SCoA hydratase/lyase may utilize caffeic acid as a substrate to form 3,4-dihydroxy benzaldehyde. This product also may be converted to vanillin through the action of the above-described 3-O-methyltransferases.

Expression vectors comprising DNA that encodes the aforementioned enzymes are introduced into a selected microorganism. Preferably, a microorganism that is amenable to genetic manipulation is utilized. In addition, it is preferred that the microorganism is not capable of degrading or further metabolizing the end product, vanillin. Suitable microorganisms for practice of the invention include, but are not limited to, *E. coli* and *Pseudomonas spp.* as model procaryotic expression systems and yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris* as model eucaryotic expression systems.

Vectors and systems for transforming these and other organisms are well known in the art.

After the microorganism has been engineered to express all enzymes necessary for the conversion of the caffeic acid or its derivatives to vanillin, production of vanillin is accomplished as follows: (1) grow the engineered microorganism in a suitable culture medium; (2) add the selected caffeic acid or derivative to the culture medium; (3) grow the culture for a time, and under conditions to enable production of vanillin, which preferably, but not necessarily, is secreted into the medium; and (4) recover the vanillin from the cells or medium. Vanillin can be purified from a solution by well-established methods (e.g., Priefert et al., 2001, Appl. Microbiol. Biotechnol. 56: 296-314; Klinke et al., 2002, Bioresource Technology 82: 15-26), used in vanillin manufacturing from lignin, for instance. Examples

include vanillin volatilization from solutions above 80°C and crystallization from saturated solutions.

The present invention also provides a novel multifunctional methyltransferase from *Vanilla planifolia*, and its encoding nucleic acid, both of which are useful in the practice of the present invention and for other purposes. This enzyme, referred to herein as “vpOMT” is capable of catalyzing the conversion of caffeic acid to ferulic acid, and also the conversion of 3,4-dihydroxybenzaldehyde to vanillin. Details of the isolation and characterization of vpOMT, and the cloning of a cDNA encoding vpOMT, are set forth in the examples.

A cDNA encoding vpOMT is set forth herein as SEQ ID NO:1, and its encoded protein is set forth herein as SEQ ID NO:2. Although this particular cDNA and polypeptide are described and exemplified herein, this invention is intended to encompass proteins from other *Vanilla* cultivars and species that are sufficiently similar to be used interchangeably with the characterized vpOMT for the purposes described herein.

Accordingly, considered in terms of their sequences, vpOMT-encoding nucleic acids of the invention include allelic variants and natural mutants of SEQ ID NO:1, which are likely to be found in different varieties of *V. planifolia* and *Vanilla*, and homologs of SEQ ID NO:1 likely to be found in different plant species. Because such variants and homologs are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated vpOMT-encoding nucleic acid molecule that encodes a vpOMT polypeptide having at least about 90% (and, with increasing order of preference, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99%) identity with SEQ ID NO:2, with a corresponding level of nucleotide sequence identity with respect to SEQ ID NO:1. Because of the natural sequence variation likely to exist among vpOMT enzymes and the genes encoding them in different plant varieties and species, one skilled in the art would expect to find this level of variation, while still maintaining the unique properties of the vpOMT of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants and homologs are considered substantially the same as one another and are included within the scope of the present invention.

VpOMT-encoding nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

VpOMT genes also may be isolated from appropriate biological sources using methods known in the art. Nucleic acids having the appropriate level sequence homology with part or all of SEQ ID NO:1 may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55°C in 2X SSC and 0.1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$$T_m = 81.5EC + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$$

As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the

hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and wash in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and wash in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and wash in 0.1X SSC and 0.5% SDS at 65 C for 15 minutes.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEM-T (Promega Biotech, Madison, WI) or pBluescript (Stratagene, La Jolla, CA), either of which is propagated in a suitable *E. coli* host cell.

VpOMT nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of SEQ ID NO:1.

VpOMT polypeptides may be prepared in a variety of ways, according to known methods. In one embodiment the protein is purified from appropriate sources, e.g., plant tissue as described in the examples.

Alternatively, the availability of nucleic acid molecules encoding the polypeptides enables production of the proteins using *in vitro* expression methods known in the art. For example, vpOMT may be produced by expression in a suitable procaryotic or eucaryotic system. Part or all of a DNA molecule, such as the cDNA having SEQ ID NO:1, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

Polyclonal or monoclonal antibodies directed toward any of the peptides encoded by vpOMT may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols.

5 The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1

Methods for Isolation and Purification of vpOMT protein and cDNA

Plant material

10 Tissue cultures of *V. planifolia* were initiated and maintained as described by Podstolski et al. (2002, *Phytochemistry* 61: 611-620). Plants of *V. planifolia* were maintained in the greenhouse and were the source of stem, leaf, and root tissues. Green *V.*
15 *planifolia* pods at different stages of development were obtained from Indonesia.

Enzyme extraction and assay

Preparation of crude protein extracts of the *V. planifolia* pods and tissue cultures grown in liquid media was modified from that described by Wang et al. (1997, *Plant Physiol.*
20 114: 213-221). For determining the presence of DOMT activity, 3 g tissue was homogenized in 6 ml of 50 mM BisTris-HCl, pH 6.9, 10 mM 2-mercaptoethanol, 5 mM Na₂S₂O₅, 1% (w/v) PVP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10% (v/v) glycerol. The homogenate was filtered through cheesecloth and centrifuged 15 min at 10,000 g at 4 °C.

Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-
25 Rad, Richmond, CA) with bovine serum albumin as a standard.

O-Methyltransferase assays were as described by Wang et al. (1997, *supra*). Assays were done in 50 µl volumes and were composed of 10 µl assay buffer (250 mM Tris-HCl, pH 7.5, 10 mM DTT), 1 µl of 50 mM substrate, 10 µl enzyme (crude extracts or fractions from partial purification), and 1 µl of S-[methyl-¹⁴C]adenosyl-L-methionine (SAM) (59
30 mCi/mmol)(Amersham Pharmacia Biotech, Buckinghamshire, England), and 28 µl water. The samples were incubated at 30 °C for 30 min, after which the reactions were stopped by adding 2.5 µl of 6 M HCl. [¹⁴C]SAM was separated from the radiolabeled methylated product by extraction with 100 µl ethyl acetate. Twenty µl of the organic phase containing the labeled product was used for liquid scintillation counting. The counts per min were

converted to pkat (picomoles of product produced per second), based on the specific activity of the substrate and the efficiency of the scintillation counter.

For determination of the kinetic parameters, the reaction conditions were modified to include 2 μ l [14 C]SAM, 100 μ M unlabelled SAM, and 3 μ g of the purified recombinant protein expressed in *E. coli*. Substrate concentrations ranged from 0.001 mM to 4 mM. All reactions were done in duplicate. Vmax and Km were calculated from nonlinear regressions of the Michaelis-Menton plots using the program Prism 4 (GraphPad Software, Inc., San Diego, CA).

Thin layer chromatography

The identity of vanillin as the labeled reaction product following methylation of 3,4-dihydroxybenzaldehyde was confirmed by TLC analysis. Twenty μ l aliquots of the organic extract were spotted onto a 20 cm x 20 cm silica gel 60 precoated TLC plate (EM Industries, Inc, Gibbstown, NJ). Twenty μ l each of 10 mM vanillin, 10 mM 3,4-dihydroxybenzaldehyde and a mixture of both were also spotted as standards. The plate was developed in a solvent system of chloroform:acetic acid (9:1, v/v). To visualize the standards following chromatography, the plate was allowed to dry and examined under UV light. The region of the plate from the reaction product that corresponded to the position of standard vanillin was scraped into scintillation vials and counted.

Partial purification of V. planifolia OMT

For protein purification, a crude extract of the tissue culture was prepared by homogenizing in 10 volumes fresh weight of the extraction buffer. Partial purification of DOMT activity from the crude extract on an adenosine-agarose affinity column was modified from that described by Wang and Pichersky (1998, Arch. Biochem, Biophys. 349:153-160). A 1 ml adenosine-agarose (Sigma, St. Louis, MO) column was prepared as previously described (Attieh et al., 1995, J. Biol. Chem. 270: 9250-9257). Ten ml of tissue culture crude extract was applied to the adenosine-agarose column. The column was washed with 6 ml 50 mM Bis-Tris, pH 6.9, 10 mM 2-mercaptoethanol, 10% glycerol followed by elution with 10 ml wash buffer containing 2.5 mM adenosine. One ml fractions were collected and assayed for DOMT and COMT activities. Fractions containing activity were combined and concentrated using Microcon YM30 devices (Amicon, Beverly, MA).

PCR amplification of O-methyltransferase cDNA fragment

Degenerate oligonucleotide primers for PCR were designed based on conserved sequences in COMTs from other plant species. The amino acid sequences encoded by the primers were VLMESWY and HVGGDMF, respectively.

5 The degenerate oligonucleotide primers were used in PCR amplification of the cDNA library prepared from the *V. planifolia* tissue cultures. PCR reactions were carried out using the Elongase Amplification System (Invitrogen, Carlsbad, CA). The 100 µl reactions contained 60 mM Tris-SO₄, pH 9.1, 18 mM (NH₄)₂SO₄, 1.5 mM MgSO₄, 200 µM each dNTP, 3 µg of each oligonucleotide, and 2 µl Elongase enzyme mix. PCR was carried out in
10 a GeneAmp 9600 thermocycler (Perkin Elmer Life Sciences, Boston, MA). Touchdown PCR cycling parameters were used. Initial denaturation was conducted at 94 °C for 30 s. Cycle 1 consisted of denaturation at 94 °C for 30 s, annealing at 66 °C for 30 s, and extension at 68 °C for 2 min. Every two subsequent cycles, the annealing temperature was decreased by 1 °C until 56 °C was reached. An additional 30 cycles at an annealing temperature of 56 °C were
15 performed, followed by a final extension at

68 °C for 10 min. PCR products were resolved on a 1.2% (w/v) agarose gel, and a single band of about 350 bp was detected. The DNA band was excised and purified using a commercial kit (QIAquick Gel Extraction Kit, Qiagen USA). The purified band was ligated into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into JM109 *E. coli*
20 competent cells. Plasmids were purified from *E. coli* transformants using a commercial kit (QIAprep Spin Miniprep Kit, Qiagen) and sequenced using SP6 and T7 primers.

cDNA library screening

A cDNA library was constructed by Stratagene (LaJolla, CA) in the λ ZAP-Express
25 vector using poly(A⁺) RNA from *V. planifolia* tissue culture. Four hundred and fifty thousand plaque-forming units were screened using the 350 bp PCR clone as probe. The cloned 350 bp fragment was labeled with [α³²P]dCTP using a commercial kit (Prime-It II Random Primer Labeling Kit, Stratagene).

The plaque lifts were prehybridized at 42 °C in 50% (v/v) formamide, 5X SSC, 5X
30 Denhardt's solution [1X Denhardt's solution is 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, 0.02% (w/v) BSA], 50 mM sodium phosphate, pH 6.8, 1% (w/v) SDS, 100 µg ml⁻¹ calf thymus DNA, and 2.5 % (w/v) dextran sulfate. The hybridization solution was 5 X 10⁵ cpm ml⁻¹ of ³²P-labeled fragment, 50% (v/v) formamide, 5X SSC, 1X Denhardt's solution, 20 mM

sodium phosphate, pH 6.8, 1% (w/v) SDS, 100 $\mu\text{g ml}^{-1}$ calf thymus DNA, and 5% (w/v) dextran sulfate. Hybridized membranes were washed with 2X SSPE, 0.5% (w/v) SDS for 15 minutes at room temperature, 2X SSPE, 0.5% (w/v) SDS for 15 minutes at 65 °C, and 0.2X SSPE, 0.2% (w/v) SDS for 15 minutes at 65 °C. The washed filters were exposed to X-Ray film (XOMAT-AR, Kodak, Rochester, NY) with an intensifying screen. Positive plaques were subjected to two additional rounds of screening to isolate single positive plaques. The cDNA inserts from positive plaques were excised from the λ -vector as recombinant pBK-CMV phagemids. A full-length clone was completely sequenced by primer walking.

Expression of the V. planifolia OMT in Escherichia coli

The coding sequence of the OMT was amplified by PCR using oligonucleotides that introduced *Xho*I sites at the 5' and 3' ends. The PCR amplification product was separated on a 1% (w/v) agarose gel and the DNA band excised from the gel and extracted using a commercial kit (QIAquick Gel Extraction Kit, Qiagen). The PCR product was digested with *Xho*I and again gel purified. The digested PCR product was then ligated to the *Xho*I-digested dephosphorylated pET-15b expression vector (Novagen, Madison, WI) and transformed into ElectroMAX™ DH10B cells (Invitrogen) via electroporation. Plasmids from positive transformants were completely sequenced to confirm that no errors had been introduced through the PCR process. A plasmid containing the perfect OMT sequence was then transformed in BL21(DE3) cells (Novagen) for protein expression.

A BL21(DE3) OMT transformant was grown at 37°C in LB with 50 $\mu\text{g ml}^{-1}$ ampicillin to $\text{OD}_{600} = 0.5$. Protein expression was then induced by adding IPTG to 0.05 mM. Additional 50 $\mu\text{g ml}^{-1}$ ampicillin was also added and the cells grown overnight at 20 °C. The cells were collected by centrifugation at 12,000g for 15 min, lysed using BugBuster™ Protein Extraction Reagent (Novogen) and treated with Benzonase Nuclease (Novogen) according to the manufacturer's instructions. Cell debris was removed by centrifugation at 12,000g for 20 min, the clarified lysate was applied to a His-Bind column (Novogen), and the expressed OMT protein eluted according to the manufacturer's instructions. The eluted protein was passed through a PD10 column (Amersham Pharmacia Biotech AB, Uppsala Sweden) equilibrated with the OMT assay buffer and concentrated 3-fold using Ultrafree-4 centrifugal filter units (Millipore Corporation, Bedford, MA). The concentrated protein was used for enzyme activity assays.

Antibody production and immunoblot analysis

The purified recombinant protein was used for preparation of *V. planifolia* OMT-specific antiserum. The purified protein was mixed with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant and was injected into the subscapular space of a rabbit. Three injections of about 100 µg of protein each were given at 4-week intervals.

For immunoblot analysis, proteins were extracted by homogenizing tissue samples in phosphate-buffered saline (1.5 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 145.5 mM NaCl) in a ratio of 0.4 g 800 µl⁻¹. The extracts were centrifuged to remove debris and the protein concentrations of the supernatants determined using the Bio-Rad protein assay reagent. Twenty µg of protein was mixed with an equal volume of 2x sodium dodecyl sulfate (SDS) sample buffer [2x: 125 mM Tris, pH 6.8, 4.6% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.002% bromophenol blue (w/v)]. The proteins were transferred to nitrocellulose membranes (NitroPure, Osmonics, Westborough, MA) in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS), pH 11, 10% methanol (v/v). Processing and detection by chemiluminescence (Western Lightening Chemiluminescence Kit, Perkin Elmer Life Science) was according to the manufacturer's instructions.

EXAMPLE 2**Characterization of vpOMT Activity, Protein and cDNA***3,4-Dihydroxybenzaldehyde-O-methyltransferase activity in V. planifolia pods and tissue culture*

A three-step pathway for vanillin biosynthesis from 4-coumaric acid has been proposed based on precursor accumulation and on feeding cell cultures of *V. planifolia* with the proposed precursors (Havkin-Frenkel et al., in: T.J. Fu, G. Singh, W.R. Curtis (Eds.), Plant Cell and Tissue Culture for the Production of Food Ingredients, Kluwer Academic Press/Plenum Publishers, New York, 1999, pp 35-43). In this pathway 4-coumaric acid is first converted to 4-hydroxybenzaldehyde through a chain-shortening step. Hydroxylation at position 3 on the ring results in 3,4-dihydroxybenzaldehyde (also called protocatechuic aldehyde). The 3-hydroxyl group is then methylated producing vanillin. An enzyme from *V. planifolia* that catalyzes the chain-shortening step, 4-hydroxybenzaldehyde synthase, has been isolated as described hereinabove (see also, Podstolski et al., 2002, *supra*).

The proposed 3-step vanillin biosynthetic pathway postulates a 3,4-dihydroxybenzaldehyde-*O*-methyltransferase (DOMT) activity as the final step resulting in the production of vanillin. Green *V. planifolia* pods at different stages of development were obtained from Indonesia. Crude extracts of the inner region of the pods where vanillin is synthesized were assayed for DOMT activity by following the transfer of [¹⁴C] from radiolabelled SAM to 3,4-dihydroxybenzaldehyde. DOMT activity doubled between 3 and 5 months after pollination and was maintained at a similar level through 11 months after pollination (Table 2). The increase in DOMT activity at 5 months after pollination corresponded to the developmental stage when vanillin accumulation in the pods begins.

Table 2. DOMT and COMT activities (pkat mg⁻¹ protein) in *V. planifolia* pods and tissue culture extracts. Values presented are the means of duplicate assays.

| Sample | DOMT Activity | COMT Activity |
|-----------------------------|-----------------------------|-------------------|
| | <i>pkat mg⁻¹</i> | |
| Pods, crude extracts | | |
| Months after pollination | | |
| 3 | 0.37 | N.D. ¹ |
| 5 | 0.90 | N.D. |
| 8 | 0.78 | N.D. |
| 11 | 0.80 | N.D. |
| Tissue culture | | |
| Crude extract | 0.96 | 0.79 |
| Adenosine column | 17.5 | 13.2 |
| ¹ Not determined | | |

Tissue cultures of *V. planifolia* have been established that accumulate vanillin and its proposed precursors, including 3,4-dihydroxybenzaldehyde (Havkin-Frenkel et al., 1996, Plant Cell Tiss Org Cult 45: 133-136). Crude extracts of the tissue cultures were found to have both DOMT and COMT activities (Table 2). With 3,4-dihydroxybenzaldehyde as the substrate, [¹⁴C]vanillin was identified as the product by co-migration with unlabeled standard vanillin on a TLC plate. Seventy-eight percent of the radioactivity present in the crude reaction product was recovered from the TLC plate at the position of authentic vanillin.

Partial purification of DOMT activity from V. planifolia tissue culture

Since the *V. planifolia* tissue cultures had DOMT activity at similar levels to the pods and were a convenient source of plant material, the first approach to characterizing the enzyme was to purify it from the tissue cultures. Affinity purification by binding to adenosine-conjugated agarose has been successful in purifying some OMTs. Both DOMT and COMT activities could be partially co-purified from the tissue culture crude extract by chromatography on an adenosine-agarose column (Table 2). SDS gel analysis of the active fractions revealed a major band at approximately 42 kD and a minor band at approximately 27 kD. COMTs from other species are in the range of 37.6-42.3 kD. The 42 kD band seen in the SDS gel of the active fractions appeared to be a single band and was likely the source of the *O*-methyltransferase activities. Peptide sequencing of the 42 kD band, however, revealed it was heterogeneous and no sequences similar to COMTs were obtained. Additional purification attempts were made using other column chromatography methods, but none were successful in separating the DOMT and COMT activities from each other.

V. planifolia O-methyltransferase cDNA clone

To test whether the DOMT activity detected in *V. planifolia* tissue cultures originated from a multifunctional methyltransferase that could methylate both 3,4-dihydroxybenzaldehyde and caffeic acid, the inventors isolated a cDNA clone based on conserved sequences in COMTs from other species for expression in *E. coli*. Degenerate oligonucleotides based on the peptide sequences VLMESWY and HVGGD MF were used in PCR of a cDNA library prepared from the *V. planifolia* tissue culture. A 350 bp amplified band was cloned whose sequence was similar to COMTs from other plants. The PCR clone was used to screen the cDNA library and a full-length clone was obtained. A 365 amino acid protein with a molecular weight of 40,659 daltons was predicted from the cDNA sequence.

Similarity of the V. planifolia OMT to other sequences

The *V. planifolia* OMT amino acid sequence is similar to COMTs reported from other plant species but the level of identity is not high to any other sequences currently in the database. COMT sequences previously reported to be from *V. planifolia* (Xue and Brodelius, 1988, Plant Physiol. Biochem. 36: 779-788) have been withdrawn from the NCBI database and now appear to actually be from *Catharanthus roseus* (Schroder et al., 2002, Phytochemistry 59: 1-8). Phylogenetic analysis comparing 19 similar methyltransferase sequences illustrates the relationship of the *V. planifolia* OMT sequence to methyltransferases

reported from other species. The amino acid sequence of the *V. planifolia* OMT shows a similar level of divergence from the other monocot OMTs as from the dicot OMTs, perhaps reflecting its phylogenetic distance from the other reported monocot COMTs. *V. planifolia* is classified in the order Asparagales, whereas the other monocot species in the COMT sequence comparison are in the order Poales.

Although there is considerable overall amino acid sequence variability among the monocot and dicot COMTs, all the residues identified from the crystal structure of the alfalfa enzyme as being involved in substrate binding or positioning (Zubieta et al., 2002, Plant Cell 14: 1265-1277) are generally well-conserved among most of the enzymes, including the *V. planifolia* OMT. The one nonconserved substrate-binding residue in the *V. planifolia* enzyme is N185 which is H183 at the corresponding position of the alfalfa enzyme. Amino acid residues at the relative position of the alfalfa substrate-binding residue I316 exhibit considerable variation among the other COMT sequences.

Two tobacco COMTs that are quite different from each other have been reported and their substrate preferences have been compared (Maury et al., 1999, Plant Physiol. 121: 215-223; Pellegrini et al., 1993, Plant Physiol. 103: 509-517). The relative substrate preferences of tobacco class I COMT were similar to those of the alfalfa enzyme whereas tobacco class II COMT had no activity against caffeic acid and 5-OH-ferulic acid, but did have activity against 3,4-dihydroxybenzaldehyde (Maury et al., 1999, *supra*). Vanillin, the product of 3,4-dihydroxybenzaldehyde methylation, has been detected in tobacco and its accumulation was 10-fold higher in a phenylalanine ammonia-lyase overexpressing cell line. The tobacco class II COMT does differ from the alfalfa sequence at 5 of the conserved substrate binding residues, suggesting these differences may relate to the observed differences in substrate preferences.

Two barley sequences that are also quite different from each other have been reported as COMTs (Lee et al., 1997, DNA Seq. 7: 357-363; Sugimoto et al., 2003, Biosci. Biotechnol. Biochem. 67: 966-972). These two sequences stand out as different from the others in amino acid sequence comparisons in that there is sequence variation at a number of the conserved substrate-binding residues, and even in some catalytic sites, suggesting a closer evaluation of the activities and substrate preferences of those enzymes may be interesting. Barley EST sequences that are more closely related to the wheat sequence and which do have the conserved substrate binding residues have been reported (GenBank Accession Numbers AL505122, AL504589, HVSMEn0023E17f, HVSMEn0007I18f, HVSMEn0023M14f, HVSSMEn0025G07f, and HVSMEn0009H02f).

Expression of V. planifolia OMT in E. coli

The protein encoded by the *V. planifolia* OMT cDNA was expressed as an N-terminal polyhistidine-tagged fusion in *E. coli* from the expression vector pET-15b and the recombinant protein purified by affinity chromatography. The expressed protein tended to rapidly accumulate in insoluble inclusion bodies, so conditions were developed using a low concentration of IPTG and low incubation temperature to allow accumulation of soluble OMT protein.

The kinetic parameters of the purified recombinant protein were determined with several phenolic and phenylpropanoid substrates (Table 3). The enzyme exhibited a preference for 5-OH-ferulic acid ethyl ester and caffeic acid ethyl ester, although these are unlikely to serve as substrates *in vivo*. Caffeoyl aldehyde and 5-OH-coniferaldehyde were preferred over 5-OH-ferulic acid, 3,4-dihydroxybenzaldehyde, or caffeic acid. In general, the relative substrate preferences for the *V. planifolia* enzyme were similar to those reported for alfalfa COMT (Parvathi et al., 2001, Plant J. 25: 193-202., which has been confirmed by down-regulation to be involved in S lignin biosynthesis (Guo et al., 2001, Plant Cell 13: 73-88). This suggests that the *V. planifolia* enzyme characterized here may also function primarily in the synthesis of lignin.

Table 3. Relative substrate preferences of *V. planifolia* recombinant OMT

| Substrates | V _{max} /K _m |
|-------------------------------|----------------------------------|
| 5-OH-Ferulic acid ethyl ester | 38.6 |
| Caffeic acid ethyl ester | 36.0 |
| Caffeoyl aldehyde | 28.4 |
| 5-OH-Coniferaldehyde | 19.7 |
| 5-OH-Ferulic acid | 9.6 |
| 3,4-Dihydroxybenzaldehyde | 2.0 |
| Caffeic acid | 1.9 |

V. planifolia OMT expression in different tissues

Expression of the *V. planifolia* OMT in different tissues was evaluated by immunoblot analysis. As expected, the OMT protein detected in the tissue samples was slightly smaller than the purified recombinant His-tagged fusion protein. The highest OMT

protein level was in the root and tissue culture samples, with a lower level in the stem sample. No immunoreactive band at the size of the OMT was detected in the leaf or pod samples.

The origin of the higher molecular weight bands observed in the stem and leaf samples is not known. The lack of an immunoreactive band in the pod tissue was unexpected and

5 surprising, since both the pods and tissue cultures synthesize vanillin and both had DOMT activity at similar levels (Table 2). These results suggest that the DOMT activities detected in these tissues originate from distinct enzymes that do not exhibit antibody cross reactivity. If this OMT is involved in the synthesis of vanillin it must be present in the pods at low levels that are not detectable by immunoblot analysis of proteins from crude extracts. Since DOMT
10 activity was detectable in the pods, however, lack of an immunoreactive protein band suggests this OMT is not the main contributor to the observed activity. Although the *V. planifolia* OMT characterized here can convert 3,4-dihydroxybenzaldehyde to vanillin *in vitro*, the kinetic parameters and the tissue localization suggest its primary function is likely to be in
15 lignin biosynthesis.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is Claimed:

1. A transgenic organism that produces vanillin when provided with caffeic acid or an esterified derivative thereof, the organism comprising expressible transgenes encoding:
 - a) a 3-O-methyltransferase that catalyzes methylation of caffeic acid to form ferulic acid; and
 - b) a chain-shortening enzyme that non-oxidatively converts ferulic acid to vanillin.
2. The organism of claim 1, which contains an endogenous esterase that hydrolyzes esters of caffeic acid.
3. The organism of claim 1, which further comprises an expressible transgene encoding an esterase that hydrolyzes esters of caffeic acid.
4. The organism of claim 1, which is a procaryote.
5. The organism of claim 4, which is *Escherichia coli* or *Pseudomonas spp.*
6. The organism of claim 1, which is a eucaryote.
7. The organism of claim 6, which is *Pichia pastoris* or *Saccharomyces cerevisiae*.
8. The organism of claim 1, wherein the 3-O-methyltransferase is from a plant source.
9. The organism of claim 8, wherein the plant source is selected from the group consisting of *Catharanthus roseus*, *Clarkia breweri*, *Coffea canephora*, *Eucalyptus gunnii*, *Festuca arundinacea*, *Hordeum vulgare*, *Lolium perenne*, *Medicago sativa*, *Nicotiana tabacum*, *Ocimum basilicum*, *Populus tremuloides*, *Prunus amygdalus*, *Saccharum officinarum*, *Sorghum bicolor*, *Thalictrum tuberosum*, *Triticum aestivum*, *Vanilla planifolia* and *Zea mays*.
10. The organism of claim 9, wherein the 3-O-methyltransferase is from *Vanilla planifolia*.
11. The organism of claim 1, wherein the chain-shortening enzyme is from a plant source.

12. The organism of claim 11, comprising a 4-hydroxybenzaldehyde synthase from *Vanilla planifolia*.

13. The organism of claim 1, wherein the chain shortening enzyme is from a bacterial source.

5 14. The organism of claim 13, comprising enoyl-SCoA hydratase/lyase.

15. A method for producing vanillin, which comprises:

a) providing a transgenic organism that produces vanillin when provided with caffeic acid or an esterified derivative thereof, the organism comprising expressible transgenes encoding:

10 i) a 3-O-methyltransferase that catalyzes methylation of caffeic acid to form ferulic acid; and

ii) a chain-shortening enzyme that non-oxidatively converts ferulic acid to vanillin;

15 b) culturing the transgenic organism in the presence of the caffeic acid or esterified derivative thereof, under conditions whereby the transgenic organism produces vanillin; and

c) recovering the vanillin from the culture.

16. The method of claim 15, wherein the organism contains an endogenous esterase that hydrolyzes esters of caffeic acid.

20 17. The method of claim 15, wherein the organism comprises an expressible transgene encoding an esterase that hydrolyzes esters of caffeic acid.

18. The method of claim 15, wherein the organism is a procaryote.

19. The method of claim 18, wherein the organism is *Escherichia coli* or *Pseudomonas spp.*

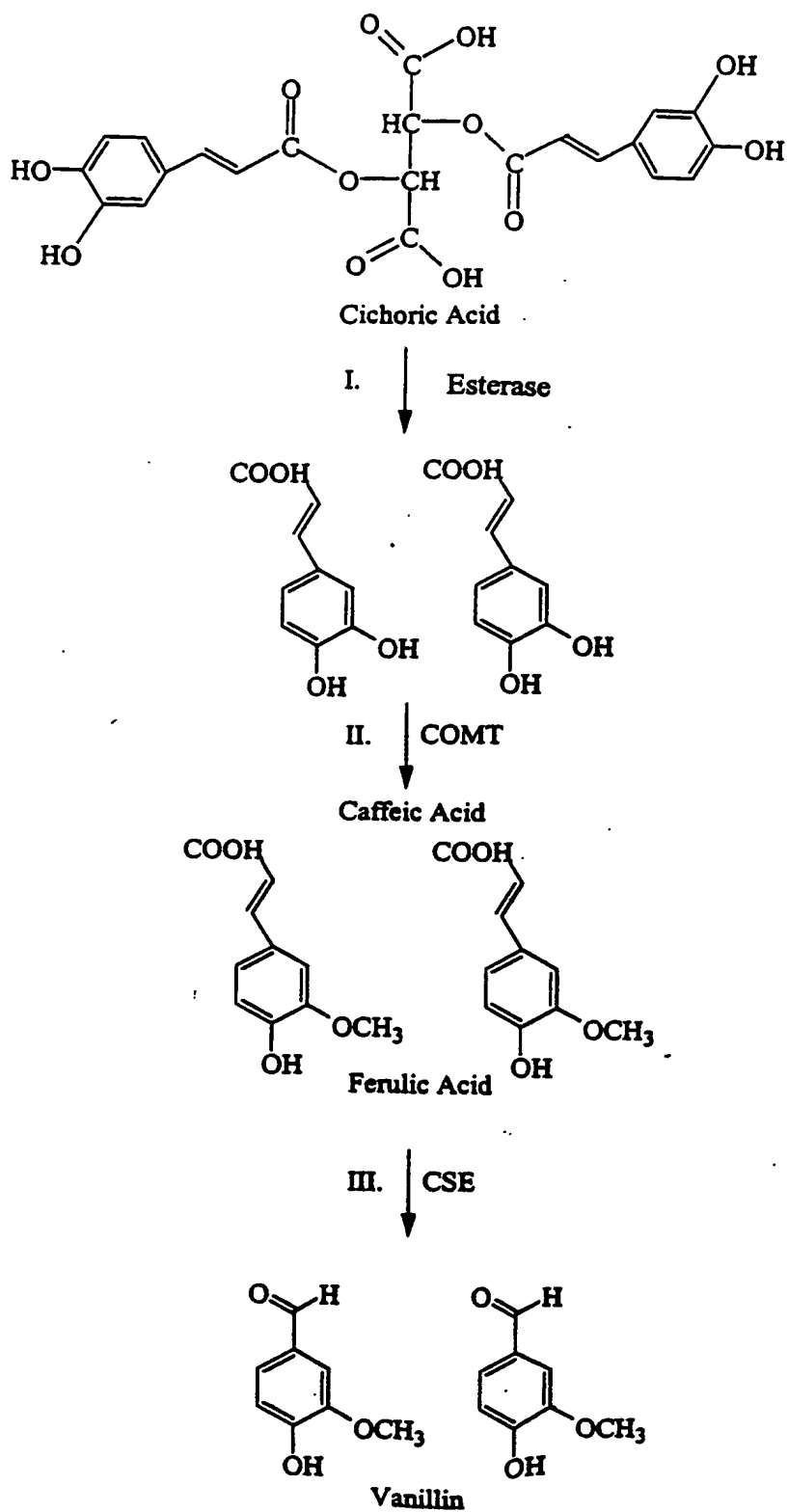
20. The method of claim 15, wherein the organism is a eucaryote.

25 21. The method of claim 20, wherein the organism is *Pichia pastoris* or *Saccharomyces cerevisiae*.

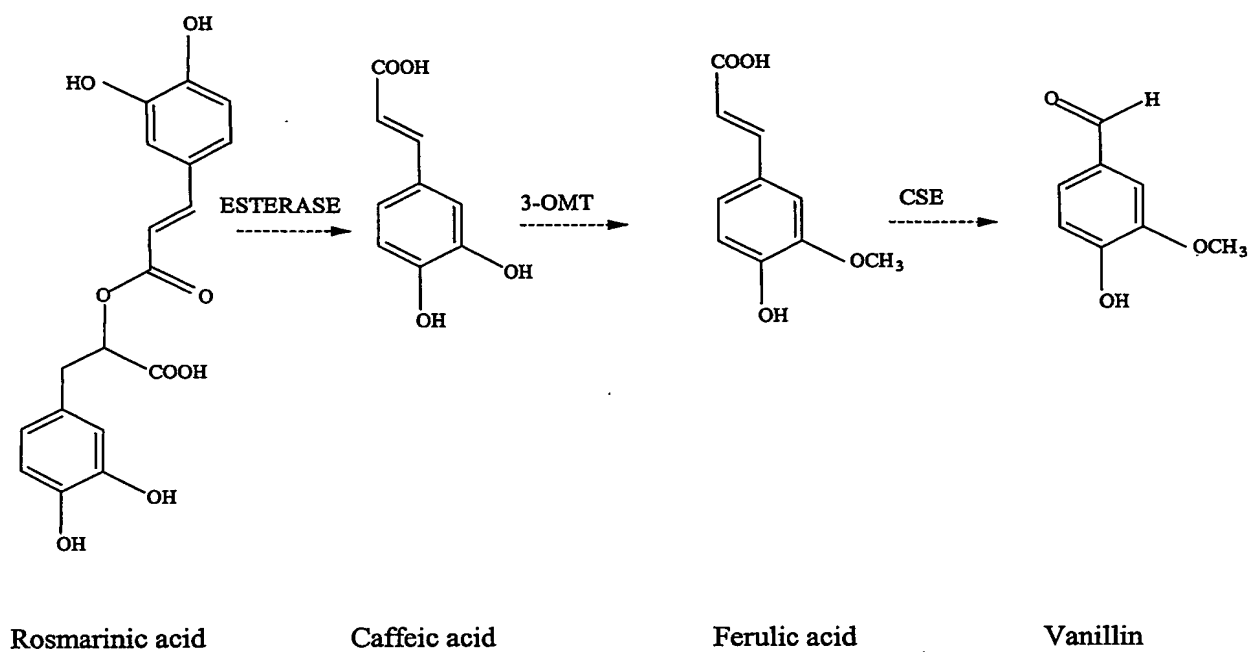
22. The method of claim 15, wherein the organism comprises a 3-O-methyltransferase from a plant source.
23. The method of claim 22, wherein the plant source is selected from the group consisting of *Catharanthus roseus*, *Clarkia breweri*, *Coffea canephora*, *Eucalyptus gunnii*, *Festuca arundinacea*, *Hordeum vulgare*, *Lolium perenne*, *Medicago sativa*, *Nicotiana tabacum*,
5 *Ocimum basilicum*, *Populus tremuloides*, *Prunus amygdalus*, *Saccharum officinarum*, *Sorghum bicolor*, *Thalictrum tuberosum*, *Triticum aestivum*, *Vanilla planifolia* and *Zea mays*.
24. The method of claim 9, wherein the organism comprises 3-O-methyltransferase from *Vanilla planifolia*.
- 10 25. The method of claim 15, wherein the organism comprises a chain-shortening enzyme from a plant source.
26. The method of claim 25, wherein the organism comprises a 4-hydroxybenzaldehyde synthase from *Vanilla planifolia*.
28. The method of claim 15, wherein the organism comprises a chain shortening enzyme
15 from a bacterial source.
29. The method of claim 28, wherein the organism comprises enoyl-SCoA hydratase/lyase.
30. The method of claim 15, comprising providing the organism with caffeic acid.
32. The method of claim 15, comprising providing the organism with a caffeic acid ester.
33. The method of claim 33, wherein the caffeic acid ester is one or more of cichoric acid, rosmarinic acid, chlorogenic acid, 1-caffeoylquinic acid or 1,5-dicaffeoylquinic acid.
20
34. An O-methyltransferase from *Vanilla planifolia* that catalyzes methylation of substrates selected from the group consisting of 5-OH-ferulic acid ethyl ester, caffeic acid ethyl ester, caffeoyl aldehyde, 5-OH-coniferaldehyde, 5-OH-ferulic acid, 3,4-dihydroxybenzaldehyde and caffeic acid.

35. The O-methyltransferase of claim 34, having an amino acid sequence at least 90% identical to SEQ ID NO:2.
36. The O-methyltransferase of claim 35, comprising amino acid SEQ ID NO:2.
37. An isolated nucleic acid molecule that encodes the O-methyltransferase of claim 34.
- 5 38. The isolated nucleic acid molecule of claim 37, which encodes a polypeptide having an amino acid sequence at least 90% identical to SEQ ID NO:2.
39. The isolated nucleic acid molecule of claim 38, which encodes a polypeptide having SEQ ID NO:2.
40. The isolated nucleic acid molecule of claim 39, having a sequence of SEQ ID NO:1.

1/2

**Figure 1**

2/2

**Figure 2**

RUCC0063.ST25.txt
SEQUENCE LISTING

<110> Rutgers, The State University of New Jersey
Havkin-Frenkel, Daphna
Zylstra, Gerben J.
Frenkel, Chaim
Belanger, Faith

<120> PRODUCTION OF VANILLIN IN MICROBIAL CELLS

<130> RUCC-0063

<150> US 60/412,649

<151> 2002-10-23

<160> 2

<170> PatentIn version 3.2

<210> 1

<211> 1219

<212> DNA

<213> Vanilla planifolia

<400> 1
gaaatggcta catgggtgga gcaccaacag cagcaaaatg gatccaagga cgtggacgag 60
gaggcgtgca tgtacgccat gcagttgtcg agcatggtcg tcctcccgat gacgcttagg 120
gtagccgtcg agctcggcat actcgaacaa atccaggccg ggggcccaaga ttcgtacctt 180
actgccgagg atttggcggc gaggtcggc aactccaacc ccttagctcc ggtcatgac 240
gagcggatcc tgcgcctgct caccagctac tccatcctta acttcaccga caccgtcgac 300
ggggagggta ggaccgtccg gagctacggc gcggcgcatg tctgcaagta cctgactccc 360
aaccaggacg gcgtctccat ggcgcctctc gtcctcatga acacggataa ggtccttatg 420
gagagctggt accacatgaa ggatgcagtg acaaatggtg gaataccatt caatctagca 480
tatgggatga cagcttttga gtatcatggg aaagatctaa ggtttaataa ggtgttcaac 540
gagggcatga agaacaactc gatcattata acgaagaaga ttttagagag atacaaaagg 600
tttgaagatg tcaatgtttt aattgatgtt ggtggtggaa ttggtggaac tatcagtatg 660
attactgcaa agtatccaca tatacatggg attaattttg accttcctca tgttgtttct 720
gaagctccac ctttccaagg ggtagaacat gtcgggtggaa acatgtttga aagtgtcccc 780
attggtgatg caatcttcat aaagtggatt cttcatgatt ggagtgatga gcattgtttg 840
aagctcctaa gaaattgtgc aaaatcttta cctgacaaag gaaaagtcac agttgtggaa 900
tgcattcttc ccgatgcacc tttggtgacg ccagaggctg aaggtgtctt tcatttggac 960
atgataatgt tggctcacia tcctggggga aaggagagaa caaagaaaga gtttaaagaa 1020
ttggctatgc tatctggttt ctctaatttc aaggcacttt ttagttatgc taatgtttgg 1080
gtcatggaat tcaacaaata gtaatcttga aactccatat tgtgtggcta atttggatgc 1140
tattttcaca tgcaaggatg tgggtgtctct tattatatga atgtatgcat tatttttctt 1200
aaaaaaaaa aaaaaaaaaa 1219

<210> 2

<211> 365

RUCC0063.ST25.txt

<212> PRT

<213> Vanilla planifolia

<400> 2

Met Ala Thr Trp Val Glu His Gln Gln Gln Gln Asn Gly Ser Lys Asp
 1 5 10 15

Val Asp Glu Glu Ala Cys Met Tyr Ala Met Gln Leu Ser Ser Met Val
 20 25 30

Val Leu Pro Met Thr Leu Arg Val Ala Val Glu Leu Gly Ile Leu Glu
 35 40 45

Gln Ile Gln Ala Gly Gly Pro Asp Ser Tyr Leu Thr Ala Glu Asp Leu
 50 55 60

Ala Ala Arg Leu Gly Asn Ser Asn Pro Leu Ala Pro Val Met Ile Glu
 65 70 75 80

Arg Ile Leu Arg Leu Leu Thr Ser Tyr Ser Ile Leu Asn Phe Thr Asp
 85 90 95

Thr Val Asp Gly Glu Gly Arg Thr Val Arg Ser Tyr Gly Ala Ala His
 100 105 110

Val Cys Lys Tyr Leu Thr Pro Asn Gln Asp Gly Val Ser Met Ala Pro
 115 120 125

Leu Val Leu Met Asn Thr Asp Lys Val Leu Met Glu Ser Trp Tyr His
 130 135 140

Met Lys Asp Ala Val Thr Asn Gly Gly Ile Pro Phe Asn Leu Ala Tyr
 145 150 155 160

Gly Met Thr Ala Phe Glu Tyr His Gly Lys Asp Leu Arg Phe Asn Lys
 165 170 175

Val Phe Asn Glu Gly Met Lys Asn Asn Ser Ile Ile Ile Thr Lys Lys
 180 185 190

Ile Leu Glu Arg Tyr Lys Arg Phe Glu Asp Val Asn Val Leu Ile Asp
 195 200 205

Val Gly Gly Gly Ile Gly Gly Thr Ile Ser Met Ile Thr Ala Lys Tyr
 210 215 220

Pro His Ile His Gly Ile Asn Phe Asp Leu Pro His Val Val Ser Glu
 225 230 235 240

Ala Pro Pro Phe Gln Gly Val Glu His Val Gly Gly Asn Met Phe Glu
 245 250 255

Ser Val Pro Ile Gly Asp Ala Ile Phe Ile Lys Trp Ile Leu His Asp

RUCC0063.ST25.txt

260

265

270

Trp Ser Asp Glu His Cys Leu Lys Leu Leu Arg Asn Cys Ala Lys Ser
275 280 285

Leu Pro Asp Lys Gly Lys Val Ile Val Val Glu Cys Ile Leu Pro Asp
290 295 300

Ala Pro Leu Val Thr Pro Glu Ala Glu Gly Val Phe His Leu Asp Met
305 310 315 320

Ile Met Leu Ala His Asn Pro Gly Gly Lys Glu Arg Thr Lys Lys Glu
325 330 335

Phe Lys Glu Leu Ala Met Leu Ser Gly Phe Ser Asn Phe Lys Ala Leu
340 345 350

Phe Ser Tyr Ala Asn Val Trp Val Met Glu Phe Asn Lys
355 360 365